

ET-18-O-CH₃-induced apoptosis is causally linked to COX-2 upregulation in H-ras transformed human breast epithelial cells

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Received 3 July 2005; accepted 10 September 2005

Available online 14 October 2005

Edited by Laszlo Nagy

Abstract Abnormally elevated expression of cyclooxygenase-2 (COX-2) has been frequently observed in transformed or malignant cells, and certain non-steroidal anti-inflammatory drugs with COX-2 inhibitory activity exert anti-neoplastic or chemopreventive effects. Contrary to this notion, we have found that a novel alkylphospholipid type antitumor agent ET-18-O-CH₃ (1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine) induces COX-2 expression in H-*ras* transformed human breast epithelial cells (MCF10A-*ras*) while it causes apoptosis at the same concentration range. The addition of a selective COX-2 inhibitor SC-58635 and COX-2 gene knock down with the siRNA blocked ET-18-O-CH₃-induced apoptosis, suggesting that COX-2 induction by this drug is causally linked to its apoptosis inducing activity. ET-18-O-CH₃ enhanced the transcriptional activities of cyclic AMP response element which is a key regulator of COX-2 expression. 15-Deoxy-Δ^{12,14} prostaglandin J₂ is, an endogenous ligand for peroxisome proliferator-activated receptor γ (PPARγ), has been known to possess proapoptotic potential in diverse cell types. ET-18-O-CH₃ treatment resulted in elevated release of 15d-PGJ₂ and DNA binding and transcriptional activity of PPARγ. Based on these findings, it is likely that ET-18-O-CH₃ induces COX-2 expression and production of 15d-PGJ₂ which may mediate the ET-18-O-CH₃-induced apoptosis in MCF10A-*ras* cells. © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Cyclooxygenase-2; Apoptosis; ET-18-O-CH₃; 15-Deoxy-Δ^{12,14}-prostaglandin J₂; Human breast epithelial cells transformed with H-*ras* (MCF10A-*ras*)

1. Introduction

Cyclooxygenase (COX) initiates the conversion of arachidonate to a series of prostaglandins (PGs) and thromboxanes. Two isoforms of COX, i.e., COX-1 and COX-2, have been identified. COX-1, which is constitutively expressed in almost all tissues, is important for the maintenance of homeostatic functions, whereas COX-2, as an inducible isozyme, is transiently upregulated by pro-inflammatory cytokines, growth factors, tumor promoters, etc. [1]. Abnormal upregulation of COX-2 has been implicated in the pathogenesis of various human malignancies. There has been substantial body of data suggesting that COX-2 overexpression provides tumor cells with survival advantage leading to resistance to apoptosis and increased invasiveness or angiogenesis [2–4]. Conversely,

selective COX-2 inhibitors have been shown to exert anti-carcinogenic activity [5,6]. Therefore, inhibition of COX-2 has been recognized as one of the most promising strategies for cancer prevention or treatment.

However, induction of COX-2 does not necessarily contribute to the cell survival or tolerance to proapoptotic insult. Thus, certain anticancer agents with pro-apoptotic activity were found to upregulate COX-2 expression in human hepatic myofibroblasts cells [7] and neuroglioma cells [8]. According to these studies, COX-2 inhibition by non-steroidal anti-inflammatory drugs (NSAID) blunted the anti-proliferative effect of these compound, suggesting that COX-2-derived PGs may be implicated in sensitizing these cells to apoptotic death. Moreover, in line with a possible proapoptotic function of COX-2, a major COX-2 product prostaglandin E₂ (PGE₂) [8] as well as 15-deoxy-Δ^{12,14}-PGJ₂ (15d-PGJ₂) [9–11], a ligand of peroxisome proliferator-activated receptor γ (PPARγ), induced apoptosis in several types of cancer cells. Nonetheless, the molecular mechanism linking upregulation of COX-2 to induction of apoptosis has not been resolved yet.

ET-18-O-CH₃ (edelfosine; 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) is a synthetic analogue of 2-lyso-phosphatidylcholine that has been found to target cellular membranes and to exert potent anti-neoplastic effects [12,13]. ET-18-O-CH₃ has a broad spectrum of anti-tumorigenic effects [14–17]. The compound has been known to be a potent inducer of apoptosis in tumor cells, especially in leukemic cells, while sparing normal cells [18–20]. Inhibition of protein kinase C, phospholipase C, phosphatidylinositol 3-kinase, CTP: phosphocholine cytidyltransferase, and coenzyme A-independent transacylase as well as blocking of arachidonate-phospholipid remodeling contributes to ET-18-O-CH₃-induced apoptosis [20–27]. ET-18-O-CH₃-induced apoptosis was accompanied by disruption of the mitochondrial transmembrane potential and activation of caspase-3 [15,28]. It also induces cell death by intracellular activation of the death receptor Fas/CD95 [18,29,30].

Here, we report that upregulation of COX-2 contributes to apoptotic death of the *ras*-transformed human mammary epithelial cells treated with the anti-cancer drug ET-18-O-CH₃.

2. Materials and methods

2.1. Materials

ET-18-O-CH₃ was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA). SC58635, a specific COX-2 inhibitor, was kindly provided by Pharmacia Korea. PGE₂ and

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15d-PGJ₂ were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Dulbecco's modified Eagle's medium (DMEM)/F-12, heat-inactivated horse serum, L-glutamine, and penicillin/streptomycin/fungicide mixture were products of Gibco-BRL (Grand Island, NY, USA). Insulin, cholera toxin, hydrocortisone, recombinant epidermal growth factor, and actin antibody were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against poly(ADP-ribose)polymerase (PARP) and COX-2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cleaved PARP antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA, USA). The ECL chemiluminescent detection reagent was purchased from Amersham Co. (Arlington Heights, IL, USA). A series of human COX-2 promoter deletion constructs ligated to luciferase gene were described previously [31]. The putative peroxisome-proliferator reactive element (PPRE) firefly luciferase reporter construct (pPPRE-Luc) was kindly provided by Dr. Kang-Yell Choi (Yonsei University, Seoul, Korea). [γ -³²P]ATP was the product of NEN Life Science (Boston, MA, USA). ET-18-O-CH₃ was dissolved in 50% ethanol. Other substances were dissolved in DMSO and was further diluted with culture medium.

2.2. Cell culture

The MCF10A cell line transfected with a virus carrying the H-ras oncogene (MCF10A-ras) was cultured as described previously [32]. The cells were cultured in DMEM/F-12 medium supplemented with 5% heat-inactivated horse serum, 10 μ g/ml insulin, 100 ng/ml cholera toxin, 0.5 μ g/ml hydrocortisone, 20 ng/ml recombinant EGF, 2 mM L-glutamine, and 100 μ g/ml penicillin/streptomycin/fungi zone mixture at 37 °C in a 5% CO₂ atmosphere.

2.3. Cell growth assay

MCF10A-ras cells at 50–60% confluence were inoculated into the plate and exposed to the medium containing chemicals. The cell viability was determined by the trypan blue exclusion method or the conventional MTT reduction assay as described previously [16,33]. All samples were prepared in triplicates.

2.4. Measurement of PGs

MCF10A-ras cells cultured in 6-well plates were treated with ET-18-O-CH₃ with or without SC58635 for 3 days. The amounts of PGE₂ released into media were measured using the enzyme-immunoassay kit (Amersham Biosciences Corp., NJ, USA) according to the manufacturer's instructions. Briefly, 50 μ l of culture medium previously centrifuged at 200 \times g for 10 min was mixed with 50 μ l PGE₂ antibody solution in the plate coated with goat anti-mouse IgG followed by incubation on ice for 3 h. After 50 μ l horseradish peroxidase conjugate PGE₂ was added to the reaction mixture, the plate was kept on ice for additional 1 h. After aspiration and rinse four times with washing buffer, 150 μ l of 3,3',5,5'-tetramethylbenzidine substrate solution was added and incubation was continued for 30 min at room temperature in a dark place. The reaction was terminated by addition of 100 μ l of 1 M sulfuric acid, and the absorbance at 450 nm was read by the ELISA reader. PGE₂ was quantitated using a standard curve constructed with known concentrations of PGE₂. Likewise, 15d-PGJ₂ was assayed using an enzyme immunoassay kit (Assay Designs Inc., Ann Arbor, MI, USA).

2.5. In situ nick end-labeling (TUNEL)

To detect apoptosis at a single cell level, enzymatic in situ nick end-labeling (TUNEL) was carried out with an in situ death detection kit (Boehringer Mannheim, Mannheim, Germany), according to a manufacturer's protocol. Briefly, MCF10A-ras cells were cultured in a chamber slide[®] in the absence or presence of ET-18-O-CH₃ for 3 days. The cells were fixed for 30 min in 10% neutral-buffered formalin solution at room temperature. Endogenous peroxidase was inactivated by incubation with 0.3% (v/v) hydrogen peroxide in methanol for 1 h at room temperature and further incubated in a permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100) for 2 min at 4 °C. The cells were labeled by incubation with the TUNEL reaction mixture for 60 min at 37 °C followed by labeling with peroxidase-conjugated anti-fluorescein anti-goat antibody (Fab fragment) for additional

30 min. After being stained with diaminobenzidine for 10 min, cells were rinsed with phosphate-buffered saline (PBS) and mounted with 50% glycerol.

2.6. Measurement of sub-diploid DNA

MCF10A-ras cells plated at a density of 2×10^5 cells in 6-well plates were treated with ET-18-O-CH₃ in the presence or absence of SC58635 for 24 h. The cells were washed, trypsinized, collected by centrifuged at 200 \times g for 5 min, fixed with 1 ml of 70% cold ethanol and stored at –20 °C until use. After centrifugation at 1300 \times g for 10 min, the fixed cells were stained with PBS containing 0.1% Triton X-100, 0.1 mM EDTA (pH 7.4), 10 μ g/ml RNase A, and 50 μ g/ml PI, and 10 000 cells per sample were analyzed by a FACScalibur instrument (Becton–Dickinson, USA). The DNA histograms were further analyzed by CellQuest Pro software to calculate the proportion of sub-diploid cell population.

2.7. Measurement of mitochondrial transmembrane potential

To measure the mitochondrial transmembrane potential ($\Delta\psi_m$), the lipophilic cationic probe TMRE was used. MCF10A-ras cells were cultured in 4 chamber slide glasses (Nunc, IL, USA). After treatment, the cells were rinsed with PBS and incubated with TMRE (150 nM) in the fresh media for 30 min at 37 °C. The cells were examined under a confocal microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) with the fluorescence excitation at 488 nm and emission at 590 nm.

2.8. Western blot analysis

Treated MCF10A-ras cells were washed with PBS and harvested after digestion with lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl, pH 7.4, 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na₃VO₄, protease inhibitor cocktail tablet). Cellular debris was removed by centrifugation at 23 000 \times g for 15 min at 4 °C. The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). After addition of sample loading buffer, proteins were electrophoresed on a 12.5% SDS–polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride membranes at 300 mA for 3 h. The membranes were blocked in 5% dried milk reconstituted in 0.1% Tween 20 in PBS (PBST). The blots were then incubated with primary antibodies (COX-2, PPAR γ , caspase-3) in 3% dried milk/PBST. The blots were washed three times with PBST, and incubated with horseradish peroxidase-conjugated secondary antibodies in 3% dried milk/PBST for 1 h. The blots were washed again three times with PBST, and immunoreactive protein complexes were detected by the ECL detection reagent according to the manufacturer's instructions and visualized with X-ray film.

2.9. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from MCF10A-ras cells using TRIzol[®] (Invitrogen, Carlsbad, CA, USA). One μ g of total RNA was reverse-transcribed with murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) at 42 °C for 50 min and 72 °C for 15 min. The cycling conditions were as follows: 3 min at 95 °C followed by 35 cycles of 95 °C, 30 s; 63 °C, 1 min; 72 °C, 1 min of COX-2; 26 cycles of 94 °C, 1 min; 63 °C, 2 min; 72 °C, 2 min of the house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) followed by a final extension at 72 °C for 10 min. The primer pairs (forward and reverse, respectively) and the size of the expected products were as follows: COX-2, 5'-CGGGATCCATGCTCGCCCGCCCTGCTGC-3' and 5'-GCTCTAGAGCCTACAGTTCAGTCGAACGTTTC-3', 1800 base pair; GAPDH, 5'-TGAAGGTCCGGTGTCAACGGATTGGC-3' and 5'-CATGTAGGCCATGAGGTCCACCAC-3', 983 base pair. Amplification products were analyzed on 1.2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

2.10. Transient transfection and the luciferase assay

MCF10A-ras cells seeded at a density of 2×10^5 /well in a 6-well dish were grown to 60–70% confluence in complete growth media. The cells were co-transfected with 2 μ g of plasmid DNA constructs and 0.5 μ g of pCMV- β galactosidase control vector with DOTAP liposomal transfection reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. After 12-h transfection, the cells were treated with ET-18-O-CH₃ for additional 6 h and then washed

with PBS and lysed in 1× reporter lysis buffer (Promega, Madison, WI). The activities of firefly luciferase in the cell lysates were measured using the luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI) by Luminoskan luminometer (Thermo Labsystems, Helsinki, Finland). β -Galactosidase activity was measured by using the commercially available assay kit (Promega, Madison, WI). The relative luciferase activity was obtained by normalizing the firefly luciferase activity against the β -galactosidase activity.

2.11. COX-2 siRNA transfection

An oligonucleotide sequence for COX-2 siRNA was selected to knock down COX-2 expression by utilizing the siRNA Target Finder software at www.invitrogen.com. The human COX-2 specific siRNA (5'-AAG GGC UCU AGU AUA AUA GGA GAG G-3') and the non-specific siRNA (5'-AAG AGG GCU CGA UUA UUA AGG AGG G-3') were provided by Invitrogen (Carlsbad, CA, USA). MCF10A-*ras* cells were transfected with an oligonucleotide sequence for COX-2 siRNA or non-specific siRNA for 24 h with DOTAP liposomal transfection reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. After 24 h transfection, the cells were treated with ET-18-O-CH₃ or vehicle alone for 6 h.

2.12. Preparation of nuclear extracts

MCF10A-*ras* cells were cultured in 100-mm dishes in the absence or presence of ET-18-O-CH₃. Cells were gently washed with cold PBS, scraped, and centrifuged at 1300 × *g* for 5 min. Pellets were suspended in cold hypotonic buffer [10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.3 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. The lysates were incubated for 10 min on ice and then centrifuged at 20200 × *g* for 15 min at 4 °C. The pellets were washed with hypotonic buffer and resuspended in hypertonic buffer (30 mM HEPES, 1.5 mM MgCl₂, 0.3 mM EDTA, 10% glycerol, 450 mM NaCl, 0.1 mM PMSF) in ice for 30 min during rocking followed by centrifugation at 20200 × *g* for 15 min. After determination of the protein concentration, the supernatant was stored at −80 °C before use.

2.13. Electrophoretic mobility shift assay

The oligonucleotides harboring the PPRE consensus sequence (5'-CCAAGGTCAAAGGT-3') were end-labeled with [γ -³²P] ATP using T4 polynucleotide kinase (Takara, Japan). The nuclear protein (7–10 μ g) was incubated with 8.5 μ l of incubation buffer (30 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.3 mM EDTA, pH 8.0, 10% glycerol), 2.5 μ l polydI-dC (0.5 μ g/ μ l), and the hypertonic buffer was added up to 20 μ l of total volume. The reaction mixture was pre-incubated at room temperature for 15 min. The labeled oligonucleotide (50000–100000 cpm) was added and incubated for 30 min at room temperature for DNA-binding reactions. To ensure the specificity of the binding, a competition experiment was carried out by adding the excess unlabeled oligonucleotide to the reaction mixture before addition of the labeled oligonucleotide. Samples were separated on the 6% acrylamide gels at 150 mA in 0.25× tris-borate-EDTA buffer. After vacuum-dried, the gel was exposed to X-ray film for autoradiography at −70 °C.

3. Results

3.1. ET-18-O-CH₃-induced apoptosis in MCF10A-*ras* cells

Treatment of MCF10A-*ras* cells with ET-18-O-CH₃ inhibited the cell growth in a concentration dependent manner (Fig. 1A). ET-18-O-CH₃ treatment resulted in distinct morphological changes including rapid blebbing of plasma membrane and nuclear disintegration that are characteristic of apoptotic cell death (Fig. 1B). Moreover, MCF10A-*ras* cells treated with ET-18-O-CH₃ exhibited proteolytic cleavage of caspase-3 and the DNA repair enzyme PARP that are typical biochemical changes frequently observed in cells undergoing apoptotic death (Fig. 1C).

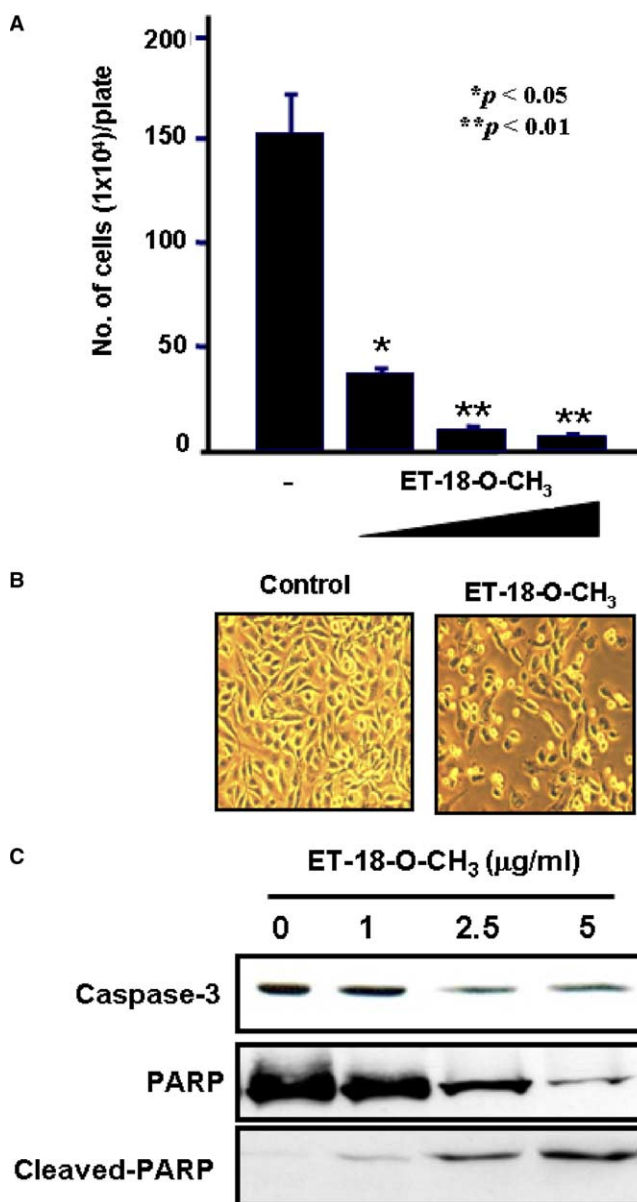


Fig. 1. Effects of ET-18-O-CH₃ on growth of MCF10A-*ras* cells. (A) The trypan blue dye exclusion method. Ten thousands of MCF10A-*ras* cells were inoculated into 35-mm dishes and exposed to the medium for 3 days with various concentrations (0, 1, 2.5, 5 μ g/ml) of ET-18-O-CH₃. Treated cells were trypsinized followed by staining with trypan blue, and viable cells were counted by a hemacytometer. Bars represent means \pm S.E.M. of triplicate experiments. A significant difference in the relative viability between treated cells and solvent controls is indicated with an asterisk. (B) Morphological changes in the MCF10A-*ras* cells treated with ET-18-O-CH₃ (2.5 μ g/ml) for 1 day. Visualized by phase-contrast microscopy. (C) ET-18-O-CH₃-induced proteolytic cleavage of caspase-3 and PARP. MCF10A-*ras* cells were exposed to indicated concentrations of ET-18-O-CH₃ for 3 days. Protein from cell lysates was resolved by SDS-PAGE by Western blot using antibodies against caspase-3, PARP, and cleaved-PARP.

3.2. ET-18-O-CH₃ upregulated COX-2 expression while inducing apoptosis

Activated *ras* oncogene has been associated with upregulation of COX-2 in breast cancer cells [34], colorectal adenomas [35,36] and non-small cell lung cancer [37]. In recognition of this notion, we attempted to determine whether ET-18-O-CH₃ could induce apoptosis through downregulation of

COX-2 expression. Contrary to our expectation, ET-18-O-CH₃-induced COX-2 expression at both protein and mRNA levels in a concentration dependent manner, whereas basal COX-2 expression in MCF10A-*ras* cells remained relatively low (Fig. 2A and B). Unlike COX-2, the expression of COX-1 was not affected by ET-18-O-CH₃ treatment in MCF10A-*ras* cells (data not shown). In parallel with elevated COX-2 expression, PGE₂ production was also significantly increased upon ET-18-O-CH₃ treatment (Fig. 2C).

3.3. Upregulation of COX-2 was causally linked to ET-18-O-CH₃-induced apoptosis in MCF10A-*ras* cells

Although upregulation of COX-2 expression has been frequently associated with resistance to apoptosis, ET-18-O-CH₃-induced apoptosis and COX-2 expression in MCF10A-*ras* cells under the same experiment conditions. To examine whether ET-18-O-CH₃-induced COX-2 expression contributes to induction of apoptosis, a selective COX-2 inhibitor SC58635 was utilized. SC58635 inhibited ET-18-O-CH₃-in-

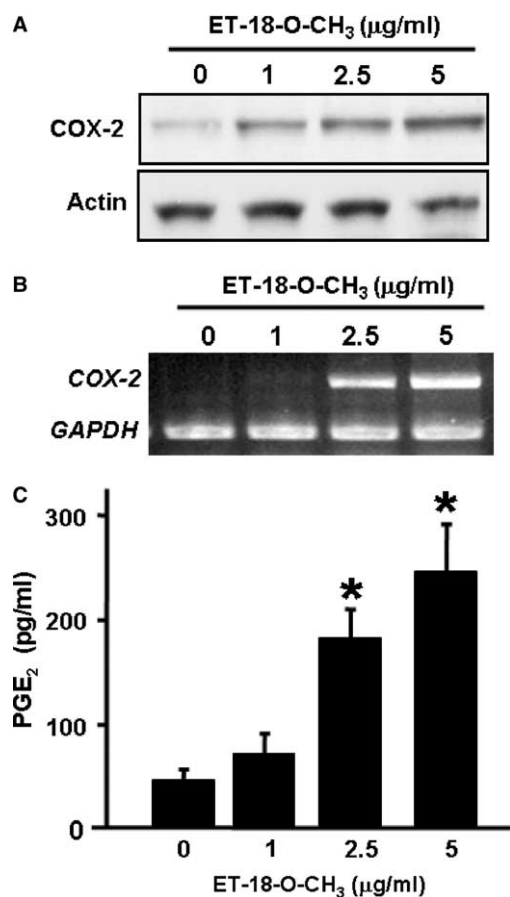


Fig. 2. Effects of ET-18-O-CH₃ on expression of COX-2 and PGE₂ production in MCF10A-*ras* cells. (A) Western blot analysis of COX-2 protein expression. MCF10A-*ras* cells were treated with various concentrations of ET-18-O-CH₃ for 3 days. Protein extracts from the cells were immunoblotted with anti-COX-2 antibody. (B) Determination of the relative amount of COX-2 mRNA. Total RNA was extracted with TRIzol[®], and RT-PCR for COX-2 mRNA transcripts was carried out as described under Section 2.9. GAPDH was used as an equal loading control. (C) ET-18-O-CH₃-induced PGE₂ production in MCF10A-*ras* cells. PGE₂ production was measured 3 days later by using the PGE₂ enzyme immunoassay kit following the manufacturer's protocol. A significant difference between treated cells and solvent controls is indicated with an asterisk ($P < 0.01$).

duced production of PGE₂ in MCF10A-*ras* cells (Fig. 3A). We observed that SC58635, at a concentration that blocks COX-2, protected MCF10A-*ras* from ET-18-O-CH₃-induced proteolytic cleavage of caspase-3 (Fig. 3B). The same concentration of SC58635 inhibited the ET-18-O-CH₃-induced DNA fragmentation and lowered the sub G₀/G₁ proportion as measured by TUNEL staining and flow cytometry, respectively (Fig. 3C). The pharmacologic inhibition of COX-2 also restored the mitochondrial transmembrane potential ($\Delta\psi_m$) which was perturbed in ET-18-O-CH₃ treated cells (Fig. 3C). Likewise, the ET-18-O-CH₃-induced PARP cleavage was abolished upon direct COX-2 gene knock down by employing the COX-2 siRNA (Fig. 3D), lending further support to the notion that upregulation of COX-2 expression is causally linked to induction of apoptosis in MCF10A-*ras* cells.

3.4. ET-18-O-CH₃ enhanced the COX-2 promoter activity

The regulation of COX-2 synthesis occurs mainly at the transcriptional level, although mRNA stabilization is also involved in response to specific signals. The types and nature of stimuli, signal transduction pathways, and transcription factors involved in the induction of COX-2 gene expression are extremely diversified and cell specific. Several *cis*-acting elements are found in the COX-2 promoter, such as nuclear factor- κ B (NF- κ B), nuclear factor-interleukin-6 (NF-IL6), cyclic AMP response element (CRE), and E-box [38,39]. In order to determine which transcription factors are involved in ET-18-O-CH₃-induced COX-2 expression, MCF10A-*ras* cells were transiently transfected with human COX-2 promoter luciferase constructs (–1432/+59) (Fig. 4A) and challenged with ET-18-O-CH₃ for 6 h. Treatment with ET-18-O-CH₃ resulted in about a 6-fold increase in the COX-2 promoter (–1432/+59) activity. To elucidate the critical region of the COX-2 promoter responsible for COX-2 expression by ET-18-O-CH₃, we utilized a series of COX-2 deletion constructs (–1432/+59, –327/+59, –220/+59, –124/+59, and –52/+59). The COX-2 promoter activity was most prominent when the –327/+59 promoter construct was used (Fig. 4B). As the promoter length was shortened, COX-2 activities were diminished gradually. It is noticeable that the –52/+59 construct exhibited an approximately 97% loss of the COX-2 promoter activity compared with the –327/+59 construct. A CRE is present between nucleotides –59 and –53, suggesting that this element might be responsible for mediating the COX-2 inducing effects of ET-18-O-CH₃. To precisely define which of these *cis*-acting elements are involved in ET-18-O-CH₃-induced COX-2 promoter activity, MCF10A-*ras* cells were transiently transfected with site-specific mutant COX-2 promoter constructs. As shown in Fig. 4C, CRM (–327/+59 construct in which mutated at the CRE site) significantly decreased the COX-2 promoter activity in ET-18-O-CH₃-treated cells. The introduction of ILM (mutated at the NF-IL6 site) resulted in approximately 2-fold reduction, compared with the wild type –327/+59 construct while little effect on COX-2 promoter activity was achieved with KBM (mutated at the NF- κ B site) (Fig. 4C). These results suggest that CRE and possibly NF-IL6 play important role in mediating the induction of COX-2 gene expression.

3.5. ET-18-O-CH₃ induced production of 15d-PGJ₂ and expression as well as activation of PPAR γ

To clarify how COX-2 upregulation by ET-18-O-CH₃ leads to induction of apoptosis in MCF10A-*ras* cells, we attempted

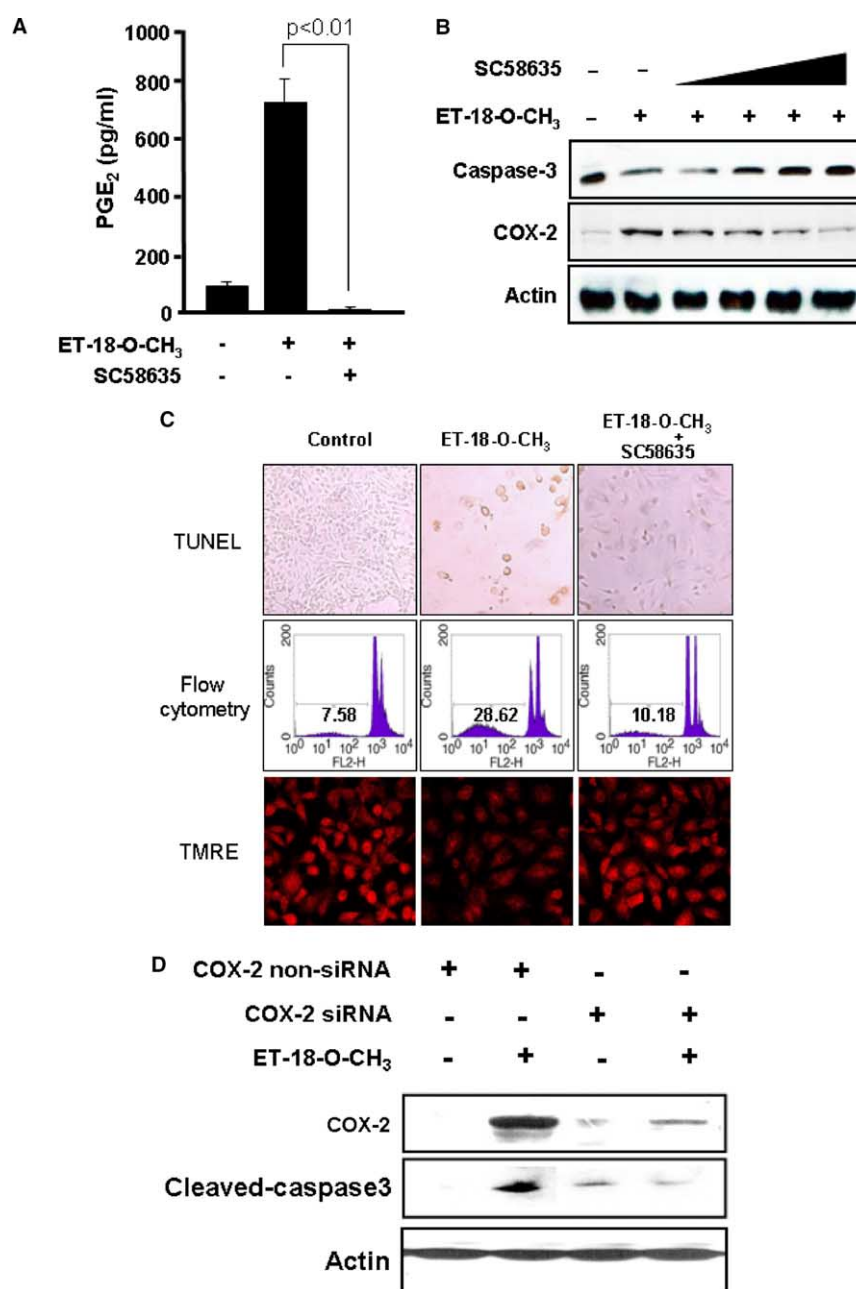


Fig. 3. Effects of the specific COX-2 inhibitor SC58635 on the ET-18-O-CH₃-induced apoptosis in MCF10A-ras cells. (A) Relative amount of PGE₂ released in the media following co-treatment of ET-18-O-CH₃ (2.5 µg/ml) for 3 days with SC58635 in the MCF10A-ras cells. (B) Effects of SC58635 on ET-18-O-CH₃-induced COX-2 expression as well as caspase-3 activation measured by Western blot analysis. MCF10A-ras cells were co-treated with ET-18-O-CH₃ (2.5 µg/ml) and 10, 25, 50 µM of SC58635 for 3 days. Protein extracts from the cells were immunoblotted with anti-COX-2, anti-caspase-3, and anti-actin antibodies. (C) SC58635 attenuation of ET-18-O-CH₃-induced apoptosis as determined by TUNEL, flow cytometry, and mitochondrial transmembrane permeability ($\Delta\psi_m$) changes. Cells were treated with ET-18-O-CH₃ (2.5 µg/ml) in the absence or presence of SC58635 (50 µM) for 1 day. (D) Effects of COX-2 siRNA on the ET-18-O-CH₃-induced COX-2 expression and cleaved PARP measured by Western blot analysis. MCF10A-ras cells were transfected with 20 nM non-specific and COX-2 specific siRNA before ET-18-O-CH₃ treatment.

to examine the products of COX-2. Among the COX-2-products, 15d-PGJ₂, an endogenous PPAR γ ligand, has been known to possess pro-apoptotic potential in diverse cancer cells [40]. To examine whether 15d-PGJ₂ can mediate ET-18-O-CH₃-induced apoptosis, we measured the production of this cyclopentenone PG in the MCF10A-ras cells treated with ET-18-O-CH₃. 15d-PGJ₂ production was significantly elevated after treatment of ET-18-O-CH₃ (Fig. 5A). Again, SC58635 suppressed the production of 15d-PGJ₂ induced by ET-18-O-

CH₃ (Fig. 5A). We also observed that exogenously added 15d-PGJ₂ inhibited the growth of MCF10A-ras cells (Fig. 5B) and induced apoptosis in MCF10A-ras cells as revealed by proteolytic cleavage of caspase-3 (Fig. 5C). As an endogenous ligand of PPAR γ , 15d-PGJ₂ is likely to exert its biologic effects in part via PPAR γ . Moreover, ET-18-O-CH₃ activated expression of PPAR γ as well as COX-2 which preceded the activation of caspase-3 through proteolytic cleavage (Fig. 5D). We also observed that ET-18-O-CH₃ induced the

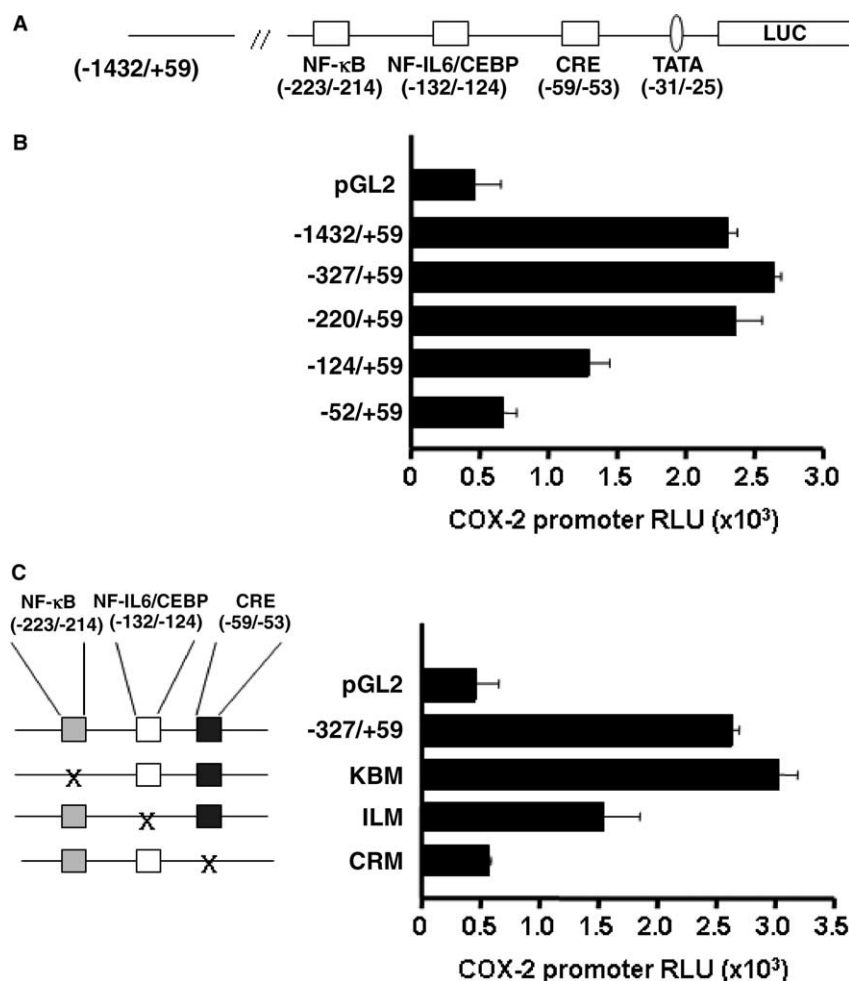


Fig. 4. ET-18-O-CH₃-induced activation of the COX-2 promoter. (A) A schematic representation of the human COX-2 promoter. (B) Determination of *cis*-acting elements of COX-2 promoter. MCF10A-*ras* cells were transfected with the 2.5 μg of a series of human COX-2 promoter deletion constructs (–1432/+59, –327/+59, –220/+59, –124/+59, –52/+59) ligated to luciferase. (C) Identification of the regions responsible for ET-18-O-CH₃-induced promoter activity of the human COX-2 gene. MCF10A-*ras* cells were transfected with 2.5 μg of a series of human COX-2 promoter-luciferase constructs (–327/+59, KBM; ILM; CRM). KBM represents the –327/+59 COX-2 promoter construct in which the NF-κB site was mutated. ILM represents the –327/+59 COX-2 promoter construct in which the NF-IL6 site was mutated. CRM refers to the –327/+59 COX-2 promoter construct in which the CRE site was mutated. For the experiments related to B and C, MCF10A-*ras* cells were transiently co-transfected with pCOX-2 promoter and pCMV-β galactosidase (0.5 μg) for 24 h by using DOTAP Liposomal Transfection Reagent according to the manufacturer's instructions. Transfectant cells were treated with ET-18-O-CH₃ (2.5 μg/ml) for 4 h and the cells were lysed with reporter lysis buffer for measurement of luciferase activity. Luciferase activity represents data that have been normalized to β-galactosidase activity.

PPRE binding activity (Fig. 5E) and transcriptional activity (Fig. 5F) of PPARγ in MCF10A-*ras* cells. Therefore, ET-18-O-CH₃-induced COX-2 expression and subsequent production of 15d-PGJ₂ are likely to contribute to induction of apoptosis in MCF10A-*ras* cells.

4. Discussion

Multiple lines of evidence indicate that aberrant overexpression of COX-2 is implicated in inhibition of apoptosis and induced proliferation [2]. Tumor formation and growth are reduced in animals that are either genetically engineered to be COX-2 deficient or treated with a pharmacologic inhibitor of COX-2 [41–43]. The use of NSAIDs has been associated with a reduced risk of several malignancies through inhibition of COX-2 activity [44,45]. Moreover intake of the selective COX-2 inhibitor celecoxib reduced the burden of colorectal

polyps in patients with familial adenomatous polyposis and has been shown to inhibit experimentally induced carcinogenesis [6,46,47]. Inhibition of COX-2 is hence recognized as one of the most feasible strategies for cancer chemoprevention and treatment.

Contrary to these findings, our present study clearly demonstrates that upregulation of COX-2 is causally linked to ET-18-O-CH₃-induced apoptosis in MCF10A-*ras* cells as a selective COX-2 inhibitor blocked the ET-18-O-CH₃-induced cell death. In this study, we found that mutation of the CRE binding site completely abolished the COX-2 promoter activity, suggesting that this site located in the COX-2 promoter plays a crucial role in regulating COX-2 transcription. Knock-down of COX-2 expression by siRNA also attenuated apoptosis induced by ET-18-O-CH₃. Other investigators have also reported that some of agents upregulate COX-2 expression while inducing apoptosis [7,48]. Thus, sphingosin 1-phosphate, a bioactive sphingolipid with growth-regulating properties,

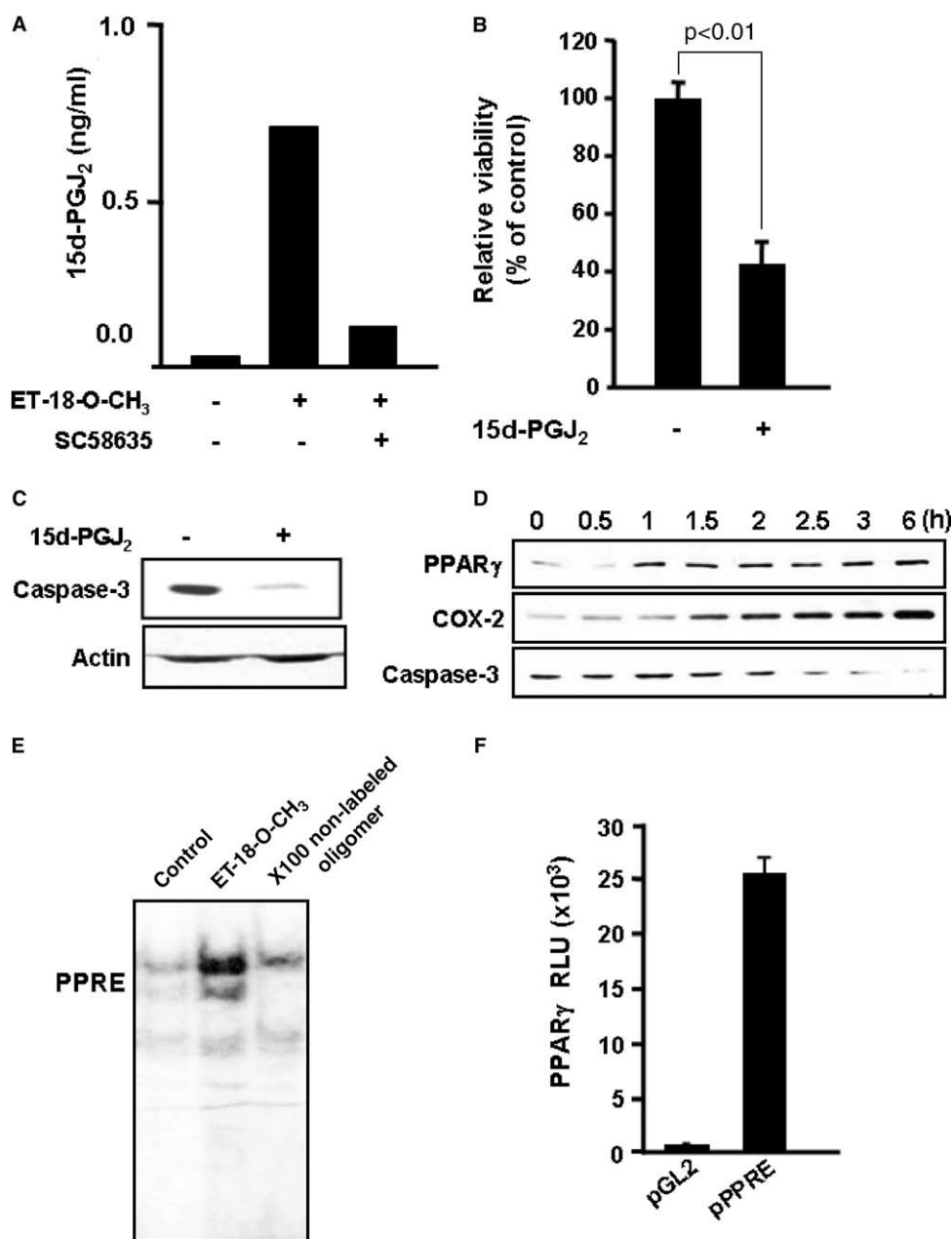


Fig. 5. Possible involvement of 15d-PGJ₂ production in ET-18-O-CH₃-induced apoptosis. (A) Production of 15d-PGJ₂ in ET-18-O-CH₃ treated MCF10A-*ras* cells. The amounts of 15d-PGJ₂ released into media were measured after treatment of the cells with 2.5 μ M of ET-18-O-CH₃ for 1 days. (B) 15d-PGJ₂ induced anti-proliferative effect in MCF10A-*ras* cells. Cell viability was measured by the conventional MTT reduction assay after treatment with 15d-PGJ₂ (10 μ M) for 1 day. Bars represent means \pm S.E.M. of triplicate experiments. (C) 15d-PGJ₂ induced apoptosis in MCF10A-*ras* cells as evidenced by caspase-3 cleavage. (D) ET-18-O-CH₃ induced expression of PPAR γ , COX-2, and caspase-3 cleavage as determined by immunoblot analysis. The concentration of ET-18-O-CH₃ was 2.5 μ M. (E) DNA-binding activity of PPAR γ in ET-18-O-CH₃ treatment MCF10A-*ras* cells. Nuclear extracts were prepared from MCF10A-*ras* cells treated with ET-18-O-CH₃ for 30 min and incubated with the [γ -³²P] labeled oligonucleotide harboring PPRE, followed by electrophoretic mobility shift assay. (F) Relative luciferase activity representing the transcriptional activity of PPAR γ . MCF10A-*ras* cells were transiently co-transfected with pPPRE-Luc and pCMV- β galactosidase for 24 h using DOTAP Liposomal Transfection Reagent according to the manufacturer's instructions. Transfectant cells were treated with ET-18-O-CH₃ (2.5 μ M) for 6 h and the cells were lysed with reporter lysis buffer for the measurement of luciferase activity.

induced COX-2 expression and apoptosis, and blocking COX-2 by NS-398 blunted the anti-proliferative effect of sphingosin 1-phosphate in human hepatic myofibroblasts cells [7]. More recently, *R*(+)-methanandamide-induced cell death has been found to be associated with COX-2 upregulation in human neuroglioma cells [8]. Some of PGs are known to have pro-apoptotic activity. For instance, 15d-PGJ₂, a potent natural ligand for PPAR γ , induces apoptosis in several types of

cancer cells [49,50]. When MCF10A-*ras* cells were treated with exogenously added 15d-PGJ₂, this cyclopentenone PG induced apoptosis in MCF10A-*ras* cells. Moreover, ET-18-O-CH₃ treatment augmented production of 15d-PGJ₂. Biological effects of 15d-PGJ₂ could be elicited via several distinct mechanisms, either PPAR γ -dependent or -independent. We found that ET-18-O-CH₃ induced expression of PPAR γ and its subsequent binding to PPRE and transcriptional activity. It has

been reported that PPRE is located in the 5' flanking region of the COX-2 promoter, and that induction of COX-2 expression by NSAIDs and PPAR γ ligands is mediated via this element [51].

Besides COX-2-dependent induction of apoptosis we demonstrated in the present work, there might be other mechanisms which can also be attributable to ET-18-O-CH $_3$ -induced apoptosis in MCF10A-*ras* cells. In this context, it is interesting to note that ET-18-O-CH $_3$ -induced apoptosis is associated with production of reactive oxygen species in p53-defective hepatocytes [15] and HL-60 cells [28].

Blockade of ET-18-O-CH $_3$ -induced apoptosis by SC58635 may have clinical implications. Recently, the chemotherapeutic agent paclitaxel-induced apoptosis in ovarian cancer cells, but combining treatment with COX-2 inhibitors resulted in a significant inhibition of paclitaxel-induced apoptosis, suggesting that combination of COX-2 inhibitors with chemotherapy agents does not have an additive or synergistic tumoricidal effect [52]. If the same effect is true in vivo, the use of COX inhibitors prior to or concurrent with anticancer drug might carry a negative effect on therapeutic efficacy. Although COX-2 selective inhibitors are obviously have chemopreventive potential, it is necessary to examine carefully any adverse effects at the whole body level before considering their application for clinical.

In summary, COX-2 upregulation contributes to induction of apoptosis in MCF10A-*ras* cells treated with the anti-cancer drug ET-18-O-CH $_3$. These findings suggest that inhibition of COX-2 expression is not necessarily desirable for cancer prevention or therapy. It seems likely that the physiological functions of COX-2 depend on the types of inducers and cells, and targeted inhibition of COX-2 in the context of anticancer therapy may need more through validation.

Acknowledgment: This work was supported by Grant R02-2004-000-10197-0 from the Basic Research Program of the Korea Research Foundation (to Y.-J. Surh).

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